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Diseases of glycosylation beyond classical congenital disorders of glycosylation

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MAJOR CONCLUSIONS: The increasing application of whole-genome sequencing techniques unravels new defects of glycosylation, which are associated to moderate forms of mental disabilities.

GENERAL SIGNIFICANCE: The knowledge gathered through the investigation of CDG increases the understanding of the functions associated to protein glycosylation in humans.

Highlights

- Diseases of glycosylation are classified as congenital disorders of glycosylation.
- CDG are rare inherited diseases with a broad range of clinical manifestation.
- To date 64 gene defects have been identified as cause of CDG.
- Genotype-phenotype comparisons reveal unexpected functional redundancies.

Keywords

Glycosylation, glycoprotein, glycolipid, glycosyltransferase, disease, mutation

1. Historical remarks

Diseases of glycosylation are rather recent additions to the growing list of known inherited diseases. In fact, most disorders of glycosylation have been described in the last 20 years. These diseases were first called carbohydrate-deficient glycoprotein syndromes (CDGS)[1] [1], but were renamed congenital disorders of glycosylation (CDG) in 1999 [2] to encompass all types of glycoconjugates. In fact, some gene defects affect multiple glycosylation pathways, hence leading to structural alteration in multiple classes of glycoconjugates. Originally, CDG have been divided into two groups. CDG-I included all disorders of N-glycosylation site occupancy and CDG-II all other disorders of N-glycosylation, O-glycosylation and glycolipid biosynthesis. As the number of glycosylation disorders approached the mark of 50, nomenclature has been simplified by focusing on the name of the mutated gene followed by the abbreviation CDG [3]. Accordingly, the disorder caused by mutations in the phosphomannomutase-2 gene is referred to as PMM2-CDG.

The initial impetus for the discovery of several diseases of glycosylation was given by the serendipitous identification of CDG cases while applying a blood test aimed at detecting alcohol abuse. In the late seventies, the neurologist Helena Stibler observed the loss of negatively charged serum transferrin in situations of chronic alcohol abuse [4]. Serum transferrin normally carries two N-glycans that are terminated by negatively-charged sialic acid (Sia). The loss of N-glycans on transferrin can easily be monitored by isoelectric focusing using few microliters of blood serum. The pediatrician Jaak Jaeken [5] and Helena Stibler [6] were the first to identify CDG patients using serum transferrin isoelectric focusing. The broad application of this simple test has paved the way to the identification of several defects of N-glycosylation. Unfortunately, similar tests unraveling defects of O-glycosylation or glycolipid glycosylation are not available, mainly because of the structural heterogeneity of O-glycans and of their tissue-specific expression. Accordingly, only few defects of O-glycosylation and glycolipid biosynthesis have been characterized so far. In fact, most of these defects were identified by genetic linkage analysis in large families.

In the last years, advanced sequencing techniques have revealed new gene defects linked to glycosylation disorders. These disorders broaden the range of symptoms and organ involvements associated with CDG. Originally, the study of severe clinical phenotypes led to the discovery of most types of CDG. However, recent developments have shown that medical teams should also consider

[1] CDGS, carbohydrate-deficient glycoprotein syndrome; CDG, congenital disorders of glycosylation; Sia, sialic acid; Dol, dolichol; Dol-P-Man, dolichol-phosphate mannose; GPI, glycosylphosphatidylinositol; Man, mannose; LLO, lipid-linked oligosaccharide; OST, oligosaccharyltransferase; ERAD, ER-associated degradation; GAG, glycosaminoglycan; PNH, paroxysmal nocturnal hemoglobinuria

milder symptoms and even additional clinical findings linked to mutations in known genes as the variability of clinical phenotypes will undoubtedly expand. In spite of the rapid progress achieved, the establishment of relationships between CDG phenotypes and specific glycoprotein and glycolipid functions remains a major challenge.

2. Defects of single and multiple glycoconjugate classes

The majority of CDG known to date are defects of N-glycosylation, which have been identified using the convenient transferrin isoelectric focusing test. Some of the gene mutations usually described in the context of N-glycosylation also affect other classes of glycosylation. For example, defects of dolichol-phosphate-mannose (Dol-P-Man) biosynthesis lead to alterations of N-glycosylation, O-mannosylation and glycosylphosphatidylinositol (GPI) assembly, because Dol-P-Man is the substrate of glycosyltransferases in these three pathways. Similarly, disorders of nucleotide-sugar transport and alterations of glycosyltransferase localization have consequences on several classes of glycosylation. In the following overview, individual gene defects are arbitrarily grouped according to the main class of glycosylation affected (Table 1).

2.1. Defects of nucleotide-sugar biosynthesis

2.1.1. Phosphomannomutase

Mannose (Man) is a major carbohydrate used in the biosynthesis of N-glycans, O-Man glycans and GPI-anchor. Accordingly, defects in the cytosolic enzymes phosphomannomutase and mannose-phosphate isomerase, which catalyze the conversion of Man-6-P to Man-1-P and of fructose-6-P to Man-6-P, respectively (Fig. 1), affect these three classes of glycosylation. Mutations in the phosphomannomutase-2 (*PMM2*, OMIM ID: 601785) gene represent by far the most frequent type of CDG, with an incidence of about 1 to 50'000. To date, more than 100 mutations in the *PMM2* gene have been described [7]. Interestingly, the R141H mutation, which is the most common mutation by far, is found in 1 out of 80 individuals in most human populations [8]. However, homozygosity for the R141H allele is never found because the R141H substitution completely abolishes the PMM2 activity, which is essential to complete embryogenesis [9]. PMM2-CDG, which was previously known as CDG-Ia, is mainly associated with neurologic symptoms like psychomotor retardation, epilepsy, ataxia and visual impairment. Other manifestations such as cardiomyopathy, hypotonia and coagulopathy are also frequently observed [10].

2.1.2. Mannose-phosphate isomerase

Whereas PMM2-CDG is mainly associated with neurological features, such a neurological involvement is missing from the clinical picture of mannose-phosphate isomerase (MPI, OMIM ID:

154550) deficiency, previously called CDG-Ib. This difference is surprising considering the proximity of the two enzymatic reactions in Man-1-P biosynthesis (Fig. 1). Most MPI-CDG cases present with diarrhea, vomiting, gastrointestinal bleeding, protein-losing enteropathy, hepatomegaly and hepatic fibrosis [11]. MPI-CDG is one of the two types of CDG that can be treated by dietary monosaccharide supplementation [12].

2.1.3. Glutamine-fructose-6-phosphate transaminase 1

Mutations in the gene encoding glutamine-fructose-6-phosphate transaminase 1 (*GFPT1*, OMIM ID: 138292) have been recently related to a form of congenital myasthenic syndrome mainly characterized by limb-girdle weakness [13]. As many as 18 GFPT1 mutations were found in 13 families of different ethnical background. The GFPT1 enzyme catalyzes a rate-limiting step in the biosynthesis of UDP-N-acetylglucosamine (GlcNAc), which is a donor substrate found in all classes of glycosylation, even counting cytoplasmic/nuclear GlcNAc-ylation [14]. UDP-GlcNAc is also a substrate in the biosynthesis of Sia, suggesting that sialylation may also be impaired in GFPT1 deficiency (Fig. 1). Decreased O-GlcNAc-ylation levels were observed in protein extracts isolated from muscle biopsies. The molecular mechanisms underlying the neuromuscular disorder are unknown. Considering the broad involvement of UDP-GlcNAc in glycosylation, it will be extremely difficult to narrow down the glycoproteins or glycolipids involved in the myasthenic phenotypes. Furthermore, intracellular UDP-GlcNAc levels fluctuate according to nutrient availability, hence affecting processes like protein O-GlcNAc-ylation and the branching of N-glycans [15]. These dynamic changes themselves influence multiple signaling pathways [16], thereby complicating even further the analysis of the functional impact of GFPT1 mutations.

2.1.4. UDP-GlcNAc 2-epimerase

As mentioned above, UDP-GlcNAc is a point of entry for the biosynthesis of Sia (Fig. 1)and the UDP-GlcNAc 2-epimerase/N-acetylmannosamine kinase (GNE, OMIM ID: 603824) enzyme is rate-limiting in this pathway [17]. Mutations in the *GNE* gene are associated with a mild form of a neuromuscular disorder called hereditary inclusion body myopathy [18]. The disease is characterized by a late onset myopathy mostly limited to leg muscles. The examination of muscle biopsies revealed intramuscular vacuoles and filamentous inclusions of unknown origin. Corresponding glycan analysis showed decreased levels of sialylated O-glycans whereas sialylation of N-glycans remained unchanged [19]. The weak phenotype of GNE deficiency raises several questions regarding the importance of Sia salvage pathways in compensating for the loss of endogenous Sia biosynthesis.

2.1.5. Galactose activation

Galactosemia, which is caused by mutations in galactokinase (*GALK*, OMIM ID: 604313), UDP-Gal 4-epimerase (*GALE*, OMIM ID: 606953) and Gal-1-P uridylyltransferase (*GALT*, OMIM ID: 606999) genes

(Fig. 1), is sometimes associated with abnormal N-glycosylation [20]. However, most symptoms result from the accumulation of toxic intermediates and by-products such as Gal-1-P and galactitol. Accordingly, galactosemia is usually not considered as a true disease of glycosylation and will not be discussed further in this overview.

2.2. Defects of dolichol biosynthesis

Some glycosyltransferase reactions require Dol-P linked donor substrates like Dol-P-Man and Dol-P-Glc. In the ER, Dol is also used as carrier for the growing oligosaccharide, which, when completed, is transferred en bloc to specific asparagine residues on nascent glycoproteins. Defects of Dol biosynthesis lead to limited availability of oligosaccharide carriers and hence to decreased N-glycosylation. To date, three defects of Dol biosynthesis have been characterized.

2.2.1. Dehydrodolichyl diphosphate synthase

Dehydrodolichyl diphosphate synthase (DHDDS, OMIM ID: 608172) is the enzyme elongating polyprenol to dehydrodolichol. The human DHDDS enzyme shares similarity to prokaryotic cis-prenyl synthases involved in undecaprenol biosynthesis. A mutation in the *DHDDS* gene, which introduces the substitution K42E, has been recently identified in a group of Ashkenazi Jewish patients affected of retinitis pigmentosa [21, 22]. These patients did not present any additional symptoms. The levels of polyprenol and the status of protein glycosylation have not been examined in DHDDS-deficient cells, so it is at this stage unclear whether the mutation has any impact on glycosylation.

2.2.2. Steroid 5-α-reductase-3

The next gene associated with decreased Dol biosynthesis is steroid 5-α-reductase-3 (*SRD5A3*, OMIM ID: 611715), which encodes the polyprenol reductase enzyme [23]. Five *SRD5A3* mutations have been reported so far in patients of Arabian, Turkish and Polish background. These mutations encompass the rearrangement 286delCAAinsTGAGTAAGGC and the nonsense mutations W107X, S10X, R142X and Y163X. These mutations lead to truncated forms of the SRD5A3 protein, suggesting that the enzymatic activity was either totally lost or strongly compromised. Despite the putative loss of polyprenol reductase activity, substantial amounts of Dol-P linked oligosaccharides were observed, thus suggesting alternate biosynthesis pathways. The patients presented with severe symptoms like mental retardation, congenital heart defects and eye anomalies, ichthyosiform dermatitis and endocrine disorders [23, 24].

2.2.3. Dolichol kinase

Dol undergoes phosphorylation before entering glycosylation pathways. This phosphorylation is catalyzed by a dedicated kinase encoded by the *TMEM15* gene (OMIM ID: 610746). To date, four patients harboring *TMEM15* mutations a have been described. The patients presented severe

symptoms like pronounced failure to thrive, ichthyosis, hypotonia, seizures, hypoglycaemia and died in early childhood [25].

Whereas glycosylation was not investigated during the characterization of the DHDDS cases, the SRD5A3 and TMEM15 deficiencies were accompanied by altered N-glycosylation as demonstrated by abnormal glycosylation of serum transferrin [23, 25]. Clinically, it is interesting to note that the DHDDS deficiency caused by the K42E substitution yielded a form of nonsyndromic retinitis pigmentosa, while the polyprenol reductase and dolichol kinase defects led to severe multi-organ dysfunctions. It is possible that the K42E substitution only slightly decreases DHDDS activity, thereby leading to a limited pathology. Alternatively, it is possible that other enzymes may replace DHDDS in polyprenol elongation or that polyprenol may be taken up or recycled from dietary sources if the rate of biosynthesis would be decreased. The identification of additional DHDDS mutations will certainly clarify this question.

2.3. Defects of lipid-linked oligosaccharide biosynthesis

After formation of Dol-P at the cytosolic side of the ER membrane, a series of glycosyltransferases add monosaccharides stepwise to complete the mature lipid-linked oligosaccharide (LLO) Dol-PP-$GlcnAc_2Man_9Glc_3$ (Fig. 2). The first glycosyltransferase along this pathway is the UDP-GlcNAc:Dol-P GlcNAc-phosphotransferase (DPAGT1, OMIM ID: 191350) that transfers GlcNAc-P to Dol-P, thereby yielding Dol-PP-GlcNAc. The subsequent cytosolically-oriented enzymes transfer GlcNAc and Man residues using the donor substrates UDP-GlcNAc and GDP-Man. After translocation of the intermediate LLO Dol-PP-$GlcNAc_2Man_5$ into the ER-lumen, other glycosyltransferases elongate LLO further using Dol-P-Man and Dol-P-Glc as substrates. Mutations have been identified in nearly all ER-glycosyltransferase genes involved in LLO biosynthesis. An extensive description of all mutations and of their effects on ER-glycosylation is provided in the recent review of Haeuptle and Hennet [7]. Clinically, alterations of LLO biosynthesis lead to broad neurological diseases of varying severity. The most frequent symptoms are psychomotor retardation, ataxia, hypotonia, seizures and coagulopathies.

Defective glycosyltransferases are not the only causes of abnormal LLO biosynthesis. Decreased availability of the substrate Dol-P-Man, such as caused by mutations in the Dol-P-Man synthase subunit genes *DPM1* (OMIM ID: 603503) [26, 27] and *DPM3* (OMIM ID: 605951) [28] (Fig. 1, 2), lead to the accumulation of the incomplete LLO and thereby to incomplete N-glycosylation site occupancy on newly synthesized glycoproteins. The MPDU1 (OMIM ID: 604041) and RFT1 (OMIM ID: 611908) proteins are also involved in LLO biosynthesis, although their exact functions remain unclear. Mutations in both genes lead to accumulation of incomplete LLO, yet through different mechanisms. The transmembraneous MPDU1 protein likely increases the accessibility of Dol-P-Man and Dol-P-Glc

substrates to the glycosyltransferases embedded in the ER membrane [29]. Accordingly, mutations in the *MDPU1* gene lead to the accumulation of Dol-PP-$GlcNAc_2Man_5$ and Dol-PP-$GlcNAc_2Man_9$ due to limited access to the corresponding donor substrates [30, 31]. The RFT1 protein facilitates the translocation of the intermediate LLO Dol-PP-$GlcNAc_2Man_5$ from the cytosolic to the luminal side of the ER membrane, possibly acting as a flippase [32]. The accumulation of the LLO Dol-PP-$GlcNAc_2Man_5$ is also typically found in cases of RFT1 deficiency [33, 34]. Mutations in the *MPDU1* and *RTF1* genes yield forms of CDG with clinical features similar to those encountered in other types of defective LLO assembly [7].

Defects leading to decreased availability of Dol-P-Man do not only impair N-glycosylation but also O-mannosylation and the biosynthesis of the GPI anchor. Along this line, deficiency of DPM1 indeed leads to decreased formation of GPI-anchored proteins, such as the complement protecting protein CD59 [26].

2.4. Defects of N-glycosylation

2.4.1. Oligosaccharyltransferase subunits

The transfer of the complete LLO Dol-PP-$GlcNAc_2Man_9Glc_3$ to nascent proteins is mediated by the oligosaccharyltransferase (OST) complex (Fig. 2), which includes at least seven subunits [35]. The *N33/TUSC3* (OMIM ID: 601385) and the *IAP/MAGT1* (OMIM ID: 300715) genes encode paralogous OST subunits that are active as oxidoreductases [36]. Mutations in these genes have been identified in few cases of nonsyndromic mental retardation [37, 38]. The impact of the *N33/TUSC3* and *IAP* mutations on OST activity is unclear. It is likely that they only affect the N-glycosylation of a restricted set of brain glycoproteins since serum glycoproteins were shown to be normally N-glycosylated in the corresponding patients [37].

2.4.2. Glucosidase I

N-glycans are first trimmed in the ER by the two glucosidase enzymes GCS1 and GCS2 (Fig. 3). A single case of glucosidase I GCS1 (OMIM ID: 601336) deficiency has been reported yet, which was linked to the compound heterozygous mutations R486T and F652L. The resulting loss of N-glycan trimming was accompanied with a severe pathology, featuring dysmorphism, hypotonia and leading to the death of the affected infant [39].

2.4.3. ER mannosidase I

The severe disease resulting from glucosidase I deficiency contrasts with another defect of N-glycan trimming, which is caused by mutations in the ER-localized mannosidase I enzyme (MAN1B1, OMIM ID: 604346). This mannosidase acts on the N-glycan core after removal of the three terminal Glc residues, thereby yielding $GlcNAc_2Man_8$ (Fig. 3). This trimming step is required for the transition of

folded glycoproteins from the ER to the Golgi apparatus. Abnormal trimming is likely to affect the elimination of unfolded glycoproteins through the ER-associated degradation (ERAD) pathway [40]. *MAN1B1* mutations have been described in twelve patients originating from five consanguineous Iranian and Pakistanis families. The impairment of these patients was limited to intellectual disabilities such as delayed speech and mild dysmorphic features [41].

2.4.4. β1-2 GlcNAc-transferase II

After trimming, N-glycans undergo elongation and branching in the Golgi apparatus (Fig. 3). A severe disease of glycosylation occurs when the branching β1-2 GlcNAc-transferase MGAT2 (OMIM ID: 602616) is affected. A deficiency of MGAT2 has only been reported in four patients, who featured severe psychomotor retardation, facial dysmorphic features, osteopenia and epilepsy among others [42, 43].

2.4.5. β1-4 Gal-transferase I

No other branching defect has been reported yet, but a defect of the elongating β1-4 Gal-transferase-1 B4GALT1 enzyme (OMIM ID: 137060) has also been associated with a form of CDG. A case of B4GALT1 deficiency has been reported by Hansske *et al.* [44], which was caused by a single base insertion resulting in the translation of a truncated B4GALT1 protein. The clinical features observed in this case of B4GALT1 deficiency were typical of CDG, showing hypotonia, Dandy-Walker malformation, coagulopathy but no psychomotor retardation. The same mutation was identified in a second patient, who presented similar symptoms, although including hepatomegaly and lacking Dandy-Walker malformation [45]. Note worthily, the B4GALT1 enzyme is also involved in the galactosylation of different classes of glycans, meaning that the phenotypes associated with B4GALT1 deficiency are not solely due to abnormal N-glycosylation (Fig. 3).

2.4.6. α2-3 Sia-transferase III

The negatively charged carbohydrate Sia is often found at the terminal position of glycan chains. The addition of Sia is catalyzed by a family of 20 sialyltransferase enzymes, which transfer Sia via α2-3, α2-6 and α2-8 linkages to various glycan acceptors [46]. The disruption of sialyltransferase genes in mice often results in immune and hematologic phenotypes [47-49]. Very recently, mutations in the α2-3 sialyltransferase gene *ST3GAL3* (OMIM ID: 606494) have been associated with nonsyndromic mental retardation [50]. The exact impact of *ST3GAL3* deficiency on brain glycoproteins remains to be established. Based on prominent expression of the *ST3GAL3* gene in skeletal muscle and considering it low expression in brain tissue [51], it can be assumed that other α2-3 sialyltransferases can compensate for the loss of ST3GAL3 activity in most tissues. Although ST3GAL3 deficiency is

discussed in the context of N-glycosylation (Fig. 3), other types of glycosylation are likely to be affected by this gene defect, too.

2.5. Defects of O-GalNAc glycosylation

2.5.1. Polypeptide GalNAc-transferase-3

Mucin type O-GalNAc glycosylation is initiated by the action of a family of polypeptide GalNAc-transferases localized in the cis-Golgi apparatus [52] (Fig. 4). Because this large family of polypeptide GalNAc-transferases ensures a functional redundancy in the O-glycosylation of protein substrates, a disease caused by mutations in a specific polypeptide GalNAc-transferase gene is difficult to predict based on clinical features. Accordingly, the association of mutations in the polypeptide GalNAc-transferase-3 gene (*GALNT3,* OMIM ID: 601756) with the disease tumoral calcinosis was surprising [53]. The primary cause of tumoral calcinosis is a deficiency of the hormone fibroblast growth factor-23 (FGF23), which regulates the calcium and phosphate metabolism [54]. FGF23 is a glycoprotein carrying O-GalNAc glycans close to a subtilisin-like proprotein convertase recognition site. The O-glycan at position Thr178 prevents the proteolytic cleavage of FGF23 and thereby its inactivation [55]. The GALNT3 enzyme is essential for the O-glycosylation of FGF23, thereby explaining the phenotypic similarity resulting from either FGF23 or GALNT3 deficiency.

2.5.2. Polypeptide GalNAc-transferase-12

Another polypeptide GalNAc-transferase isoform has been implied in the etiology of colon cancer. Heterozygous mutations in the polypeptide GalNAc-transferase-12 gene (*GALNT12,* OMIM ID: 610290) (Fig. 4) have been reported in several individuals with colon cancer [56]. Some of the mutations were detected in the germline of the patients, whereas other mutations were somatic and localized to the tumors themselves. Although it is unclear whether the decreased GALNT12 activity affects O-glycosylation in colon tissue, increased antibody reactivity towards unglycosylated MUC1 mucin protein was found in several colon tumors [56].

2.5.3. Core 1 β1-3 Gal-transferase

In contrast to the large family of polypeptide GalNAc-transferases, a single core 1 β1-3Gal-transferase catalyzes the extension of O-GalNAc (Fig. 4). The core 1 β1-3Gal-transferase enzyme is also unique among Golgi glycosyltransferases in respect to its folding. In fact, the core 1 β1-3Gal-transferase protein requires a specific chaperone called COSMC (OMIM ID: 300611), which interacts with the core 1 β1-3Gal-transferase polypeptide in the ER [57]. Mutations in the human *COSMC* gene have been reported to cause the Tn-syndrome [58], a mild hematological disorder characterized by a mosaic presentation of bare GalNAc, also called Tn-antigen, on blood cells. Such Tn-positive cells arise from hemopoietic stem cells harboring a mutated Cosmc gene and lacking core 1 β1-3Gal-

transferase activity. Persons with a Tn-syndrome are healthy and are only affected by a mild hemolytic anemia and decreased platelet and leukocyte counts.

2.6. Defects of O-Man glycosylation

Defects of O-mannosylation belong to dystroglycanopathies, since α-dystroglycan is the main carrier of O-Man chains in vertebrates. α-Dystroglycan carries multiple O-Man and O-GalNAc glycans, which contribute to the binding of α-dystroglycan to extracellular matrix proteins such as laminin-2. The clinical hallmark of dystroglycanopathies are pathology of the muscle, eye and brain [59]. The first disease associated with O-mannosylation was reported 1998, when mutations in the putative glycosyltransferase Fukutin were identified as a cause of Fukuyama-type congenital muscular dystrophy [60]. Other forms of congenital muscular dystrophies were later associated with additional glycosyltransferases involved in O-mannosylation. To date, deficiency in POMT1 (OMIM ID: 607423), POMT2 (OMIM ID: 607439), POMGNT1 (OMIM ID: 606822), LARGE (OMIM ID: 603590), Fukutin (OMIM ID: 607440) and Fukutin-related protein (FKRP) (OMIM ID: 606596) (Fig. 4) have been associated with the Walker-Warburg syndrome, Muscle-Eye-Brain disease, Fukuyama-type congenital muscular dystrophy and Limb Girdle muscular dystrophy. Mutations in the core glycosyltransferase genes *POMT1* and *POMT2* are mainly found in the most severe forms of congenital muscular dystrophies such as the Walker-Warburg syndrome [61, 62]. However, the severity of the disease depends more on the level of residual activity of the enzymes than on the position of the mutated gene along the glycosylation pathway. Accordingly, mutations in all glycosyltransferase genes involved in O-Man glycan biosynthesis have been associated with severe forms of congenital muscular dystrophy. To date, no enzymatic activity could be demonstrated for the putative glycosyltransferases Fukutin and FKRP. In addition to its role in muscle cell adhesion, α-dystroglycan is also involved in the migration of cortical neurons [63] and in retinal development [64]. The dystroglycan-laminin complex is important for the formation of the glia limitans, which prevents neurons to migrate into the subarachnoid space. Accordingly, under conditions of abnormal α-dystroglycan glycosylation, neurons overshoot across the marginal zone, thereby leading to a condition referred to as “cobblestone” lissencephaly [65].

2.7. Defects of O-Fuc glycosylation

2.7.1. Lunatic fringe

O-Fucosylation is found specifically on epidermal growth factor (EGF)-like domains and thrombospondin type 1 (TSP1) domains [66]. The transfer of Fuc to selected serine and threonine residues on EGF-like and TSP1 domains takes place in the ER and uses GDP-Fuc as donor substrate. EGF-like domains are often found repeated on several proteins. For example, the Notch receptor has

36 EGF-like domains and its ligands Delta1 and Jagged1 have respectively 8 and 16 EGF-like domains [67]. Other proteins like coagulation factors and signaling proteins also contain EGF-like domains that are likely to be O-fucosylated. The role of O-fucosylation in mammalian development is underlined by the skeletal defects resulting from abnormal vertebral segmentations. Accordingly, mutations in the lunatic fringe gene (*LFNG*, OMIM ID: 602576) (Fig. 4) encoding a β1-3 GlcNAc-transferase elongating O-Fuc have been described in cases of spondylocostal dysostosis [68].

2.7.2. β1-3 Glc-transferase

Another disease of O-fucosylation, the Peters-Plus syndrome, is caused by mutations of the β1-3 Glc-transferase gene (*B3GALTL*, OMIM ID: 610308) acting on TSP1-linked O-Fuc [69] (Fig. 4). The disease is characterized by eye chamber defects, developmental delay and growth impairment. Patients have short stature and usually do not exceed 1.5 m. A typical feature of Peters-Plus syndrome is keratolenticular adhesion, which is a caused by a developmental defect of the lens and anterior eye chamber [70]. However, the O-fucosylated glycoprotein involved in this abnormal development and the mechanisms underlying the defect are still unknown.

2.8. Defects of glycosaminoglycan biosynthesis

2.8.1. Xyl β1-4 Gal-transferase

The core structure of glycosaminoglycan (GAG) chains is unique among glycans featuring a Xyl residue β-linked to serine. The GAG core also includes two Gal units and a GlcA unit. The first Gal residue is added to Xyl by the β1-4 Gal-transferase XGPT1 (OMIM ID: 604327) (Fig. 5), which is defective in the progeroid form of the Ehlers-Danlos syndrome [71, 72]. This disease is characterized by mental retardation and connective tissues abnormalities such as loose skin, osteopenia and joint hypermobility. Only few cases of the disease have been reported to date.

2.8.2. β1-3 GlcA-transferase

Another defect of GAG core biosynthesis has been described recently. A mutation in the β1-3 GlcA-transferase gene (*B3GAT3*, OMIM ID: 606374) (Fig. 5) was found in patients presenting with short stature, congenital heart defects and joint dislocations [73]. The mutant B3GAT3 enzyme showed decreased activity, which reduced the level of heparan, chondroitin and dermatan sulfate chains on proteoglycans. Some proteoglycans like decorin were even secreted devoid of its single dermatan sulfate chain. The disease in question was also previously known as autosomal recessive Larsen-like syndrome [74].

2.8.3. EXT1/EXT2 transferase

So far, defects of GAG chain polymerization have also been associated with two diseases. The first one is called multiple hereditary exostoses and is characterized by protrusions of bone material

mainly emerging at the end of long bones and originating from benign cartilage tumors. Multiple hereditary exostoses is an autosomal dominant disease caused by heterozygous mutations in the *EXT1* (OMIM ID: 608177) and *EXT2* (OMIM ID: 608210) genes [75]. The EXT1 and EXT2 proteins form an oligomeric complex that mediates the polymerization of heparan sulfate chains [76] (Fig. 5). The inclusion of a mutant EXT1 or EXT2 subunit to the complex decreases the polymerase activity and leads to a significant reduction of heparan sulfate chains in tissues, in which the amount of such chains is critical, such as in bones. Multiple hereditary exostoses is a rather frequent condition with an incidence of 1 in 50,000 individuals.

2.8.4. Chondroitin synthase-1

The second disease of GAG chain polymerization is called Temtamy preaxial brachydactyly syndrome [77], which is caused by mutations in the chondroitin synthase-1 gene (*CHSY1*, OMIM ID: 608183). The chondroitin synthase-1 enzyme contains two catalytic domains with β1-3 GlcA-transferase and β1-4 GalNAc-transferase activities (Fig. 5). Most CHSY1 mutations identified in patients with Temtamy preaxial brachydactyly syndrome are deletions leading to truncated proteins and probably complete loss of enzymatic activity [77]. The clinical picture of the syndrome is characterized by a form of digit malformation called preaxial brachydactyly but also by mental and growth retardation, facial dysmorphism and hearing loss. The loss of CHSY1 activity in Temtamy preaxial brachydactyly syndrome suggests that additional chondroitin synthase isoforms, such as CHSY2 [78] and CHSY3 [79], are likely to compensate for the inactive CHSY1 enzyme.

2.8.5. Sulfotransferases

GAG chains are extensively modified by sulfation. Three diseases are associated with defective sulfation of carbohydrates in GAG chains. The first disease is Omani type spondylepiphyseal dysplasia [80], which is caused by a deficiency of the chondroitin 6-O sulfotransferase enzyme CHST3 (OMIM ID: 603799) catalyzing the 6-O sulfation of GalNAc found in chondroitin and dermatan sulfate chains. The symptoms of Omani type spondylepiphyseal dysplasia are typical of connective tissue disorders with short stature, skeletal dysplasia, kyphoscoliosis and arthritic joints. Mutations in the dermatan 4-sulfotransferase-1 *CHST14* gene (OMIM ID: 608429) lead to the musculocontractural type of Ehlers-Danlos syndrome [81], which is characterized by severe manifestations such as craniofacial dysmorphism, kyphoscoliosis, hypotonia and joint hypermobility. The CHST14 enzyme transfers sulfate to the 4-O position of GalNAc residues flanked on both sides by IdoA. This strict acceptor recognition explains why this sulfotransferase is specific for dermatan sulfate and does not affect chondroitin sulfate chains. The third sulfation defect affects the GlcNAc-6-sulfotransferase *CHST6* gene (OMIM ID: 605294), which is involved in the sulfation of keratan sulfate chains. The *CHST6* gene is expressed in many cell types but the clinical features of GlcNAc-6-O sulfotransferase deficiency are

limited to the eye, resulting in a progressive opacity of the cornea, a condition referred to as macular corneal dystrophy [82].

2.9. Defects of glycolipid biosynthesis

2.9.1. GM3 synthase

Only one defect of glycosphingolipid biosynthesis has been described to date. A form of infantile epilepsy occurring in the Amish community has been linked to a deficiency of the ganglioside GM3 caused by mutations in the α2-3 sialyltransferase *ST3GAL5* gene (OMIM ID: 604402) (Fig. 6). The loss of GM3 is related to symptoms like failure to thrive, psychomotor retardation, hypotonia, epilepsy, and blindness [83].

2.9.2. PIGA protein

Three gene defects are known to impair the assembly of the GPI anchor. The PIGA gene encodes a subunit of the complex initiating GPI anchor biosynthesis (Fig. 6). Somatic mutations in the X-linked *PIGA* gene (OMIM ID: 311770) arising in hemopoietic stem cells lead to the hemolytic anemia paroxysmal nocturnal hemoglobinuria (PNH) [84]. The absence of GPI-anchored proteins renders red blood cells sensitive to complement-mediated lysis, which explains the increased hemoglobin levels in the urine. PHN is also associated with increased incidence of leukemia and increased thrombotic episodes [85].

2.9.3. PIGM protein

A hypomorphic mutation in the promoter of *PIGM* mannosyltransferase gene (OMIM ID: 610273) has been also identified as a cause of low GPI expression. The PIGM enzyme transfers the first Man residue to the growing GPI core in the ER lumen [86] (Fig. 6). To date, three cases of PIGM deficiency originating from two families have been described [87]. The patients showed increased thrombosis but no signs of hemolysis, although the levels of several GPI anchored proteins were reduced on hematopoietic cells. Besides the hematologic presentation, absence seizures were also observed in the affected children.

2.9.4. PIGV protein

The PIGV gene (OMIM ID: 610274) encodes the ER-localized mannosyltransferase adding the second mannose to the GPI core [88]. PIGV deficiency is associated with a form of hyperphosphatasia and severe mental retardation, which is also referred to as Mabry syndrome [89]. Five missense mutations leading to amino acid substitutions in conserved residues of the PIGV enzyme have been identified in nine patients so far [90, 91]. Elevated serum alkaline phosphatase levels are also typically found in Mabry syndrome. This feature results from the secretion of the alkaline phosphatase enzyme that is normally anchored to the cell surface through GPI [92].

2.9.5. PIGN protein

A defect in the addition of the first ethanolamine phosphate group to GPI has been recently associated with the multiple congenital anomalies-hypotonia-seizures syndrome [93]. The GPI ethanolamine phosphate transferase-1 enzyme is encoded by the *PIGN* gene (OMIM ID: 606097) (Fig. 6), in which the homozygous mutation 2126G>A was identified in seven patients from a consanguineous Middle-eastern family [93]. The patients presented with severe neurological alterations, seizures, dysmorphic features and multiple organ impairments leading to infantile lethality.

2.10. Defects of nucleotide-sugar transport

2.10.1. GDP-Fuc transporter

Disorders of nucleotide-sugar transporters impact on multiple classes of glycosylation. Three defects of nucleotide-sugar transport have been described to date. The first one was related to mutations in the Golgi GDP-Fuc transporter gene (*SLC35C1*, OMIM ID: 605881), which leads to decreased protein fucosylation. Such fucosylated proteins are essential for the trafficking of leukocytes through the body and decreased fucosylation leads to impaired leukocyte extravasation and thus to a form of immunodeficiency called leukocyte adhesion deficiency type II (LAD2) or CDG-IIc [94]. In addition to immunodeficiency, the affected patients show short stature, mild dysmorphism and psychomotor retardation. Considering the residual transporter activity enabled by most mutations in the Golgi GDP-Fuc transporter gene, the fucosylation defect and the resulting immune disorder can be corrected by dietary fucose supplementation [95].

2.10.2. CMP-Sia transporter

Mutations in the CMP-Sia transporter gene (*SLC35A1*, OMIM ID: 605634) have only been found in a single infant patient presenting with a severe bleeding disorder designated SLC35A1-CDG or CDG-IIf [96]. The patient was compound heterozygous for two inactive *SLC35A1* alleles, which resulted in decreased protein sialylation on hematopoietic cells, whereas several serum proteins, like transferrin, were surprisingly unaffected.

2.10.3. UDP-GlcA/GalNAc transporter

Some transporters mediate the transport of two or more nucleotide sugars. The *SLC35D1* gene (OMIM ID: 610804) encodes such a multi-specific transporter, which mediates the transfer of both UDP-GlcA and UDP-GalNAc into the ER and Golgi apparatus [97]. Both substrates are critical for the biosynthesis of chondroitin sulfate chains, as demonstrated by the reduction of chondroitin sulfate on proteoglycans isolated from *SLC35D1*-null mice [98]. Mutations in the SLC35D1 gene have also been reported in humans, namely in cases of Schneckenbecken dysplasia (OMIM 269250). The

disease is characterized by severe skeletal abnormalities caused by altered cartilage and bone formation. Schneckenbecken dysplasia leads to neonatal lethality, which is also frequently observed in *SLC35D1*-null mice. Seven allelic variants of the *SLC35D1* gene have been identified in Schneckenbecken dysplasia so far [99].

2.11. Defects of vesicular transport

2.11.1. COG complex

The proper localization of glycosyltransferases and nucleotide-sugar transporters is required for normal glycosylation. Several tethering factors and vesicular transport proteins are involved in the regulation of glycosyltransferase localization [100]. Accordingly, the defective assembly of the Conserved Oligomeric Golgi (COG) complex leads to mislocalization of several glycosyltransferases and thereby to defects in multiple classes of glycosylation [101]. To date, mutations have been found in five of the eight subunit genes of the complex, namely in the COG1 (OMIM ID: 606973) [102], COG4 (OMIM ID: 606976) [103], COG5 (OMIM ID: 606821) [104], COG7 (OMIM ID: 606978) [105] and COG8 (OMIM ID: 606979) subunits [106]. The clinical pictures of COG defects are variable but in general similar to those of other types of CDG with mainly neuromuscular involvements, dysmorphia, psychomotor retardation and coagulopathy [107].

2.11.2. SEC23B protein

Congenital dyserythropoietic anemia type II (CDAN2), also called hereditary erythroblastic multinuclearity with a positive acidified serum (HEMPAS), was promptly recognized to be associated with abnormal N-glycosylation [108]. First shown to be related to low β1-2 GlcNAc-transferase II activity [109] and later to low a-mannosidase-II expression [110], CDAN2 has finally been mapped to the *SEC23B* gene (OMIM ID: 610512) [111]. The SEC23B protein is part of the coat protein complex COPII, which transport proteins from the ER to the Golgi apparatus.

2.11.3. ATP6V0A2 protein

A combined defect of N-glycosylation and O-glycosylation was recognized in a form of cutis laxa characterized by wrinkled skin, osteopenia, facial dysmorphism, hypotonia and developmental delay [112]. Using linkage analysis, the defect was localized to the *ATP6V0A2* gene (OMIM ID: 611716), which encodes a subunit of a Golgi H^+-ATPase pump possibly involved in pH regulation along the secretory pathway [113]. It is presently unclear whether the putative pH shift affects the localization or the activity of Golgi resident glycosyltransferases.

3. Conclusion

The simple analysis of serum transferrin by isoelectric focusing led to the discovery of a large family of N-glycosylation disorders. Because adequate biomarkers are not available for other classes of glycosylation, defects of O-glycosylation, GAG and glycolipid biosynthesis have long remained confined to few examples mainly identified by genetic linkage analysis. The emergence of new genome sequencing techniques will definitively boost the pace of discovery and unravel new genetic defects affecting glycosylation. In spite of this advancement, the challenge will still be to understand the mechanisms underlying the impact of gene defects on glycosylation. Whereas defective glycosyltransferases are relatively easily associated with specific glycosylation pathways, the effect of vesicular transport proteins and signaling proteins will require more work to be addressed. Nevertheless, the knowledge gathered through the investigation of diseases of glycosylation definitively improves the comprehension of glycosylation pathways and of their complex regulations.

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5. References

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Figure legends

Figure 1. Biosynthesis of the activated sugars UDP-Gal, UDP-Glc, Dol-P-Glc, UDP-GlcNAc, GDP-Man, Dol-P-Man and CMP-Sia. Sugars are represented graphically using the symbols recommended by the Consortium for Functional Glycomics (see http://glycomics.scripps.edu/CFGnomenclature.pdf). The gene defects known to impair nucleotide-sugar and Dol-P-sugar biosynthesis are marked in red beside the involved reactions.

Figure 2. Lipid-linked oligosaccharide biosynthesis. The sequential assembly of the lipid-linked oligosaccharide Dol-PP-$GlcNAc_2Man_9Glc_3$ at the ER membrane is represented with the known gene defects marked in red. The glycosyltransferases not yet associated with a type of CDG are marked in blue. The symbol OST is used to represent the complex including the subunits STT3A/B, RPN1, RPN2, DDOST, DAD1 and OST4.

Figure 3. N-glycan structures. The high-mannose form found in the ER and a typical biantennary form of N-glycans built in the Golgi apparatus are represented schematically featuring the enzymes known to be associated with CDG.

Figure 4. O-glycan structures. Typical O-GalNAc, O-Man and O-Fuc glycans are shown together with the glycosyltransferases and putative glycosyltransferases involved in some types of CDG.

Figure 5. Glycosaminogycan structures. Schematic representation of heparan, chondroitin and dermatan sulfate chains including the glycosyltransferases involved in CDG. The sulfotransferases CHST3 and CHST14, which modify chondroitin and dermatan are not shown on this figure.

Figure 6. Glycolipid structures. The ganglioside GM1 is shown as example with indication of the sialyltransferase reaction mediated by the ST3GAL5 enzyme. The structure of the GPI-anchor is represented schematically together with the enzymes defective in four types of CDG. EtN, ethanolamine.

Table I. Diseases of glycosylation			
Gene	**Gene locus MIM #**	**Function**	**Disease**
Nucleotide-sugar biosynthesis			
PMM2	601785	Phosphomannomutase (Man-6-P → Man-1-P)	PMM2-CDG (CDG-Ia)
MPI	154550	Phosphomannose isomerase (Fru-6-P → Man-6-P)	MPI-CDG (CDG-Ib)
GFPT1	138292	Glutamine:fructose-6-phosphate amidotransferase	Congenital myasthenic syndrome
GNE	603824	UDP-N-acetylglucosamine 2-epimerase	Hereditary inclusion body myopathy
GALK	604313	Gal kinase	Galactosemia
GALT	606999	Gal-1-P uridylyltransferase	Galactosemia
GALE	606953	UDP-Gal 4-epimerase	Galactosemia
Dolichol biosynthesis			
DHDDS	608172	Cis-prenol synthase	Retinitis pigmentosa
SRD5A3	611715	Polyprenol reductase	SRD5A3-CDG (CDG-Iq)
TMEM15	610746	Dol kinase	TMEM15-CDG (CDG-Im)
Lipid-linked oligosaccharide biosynthesis			
DPAGT1	191350	GlcNAc-1-P transferase	DPAGT1-CDG (CDG-Ij)
ALG1	605907	β1-4 Man-transferase	ALG1-CDG (CDG-Ik)
ALG2	607905	α1-3/6 Man-transferase	ALG2-CDG (CDG-Ii)
ALG11	613666	α1-2 Man-transferase	ALG11-CDG (CDG-Ip)
RFT1	611908	LLO translocation	RFT1-CDG (CDG-In)
MPDU1	604041	Dol-P-Man/Glc availability (?)	MPDU1-CDG (CDG-If)
DPM1	603503	Dol-P-Man synthase	DPM1-CDG (CDG-Ie)

DPM3	605951	Dol-P-Man synthase	DPM3-CDG (CDG-Io)
ALG3	608750	α1-3 Man-transferase	ALG3-CDG (CDG-Id)
ALG9	606941	α1-2 Man-transferase	ALG9-CDG (CDG-IL)
ALG12	607144	α1-6 Man-transferase	ALG12-CDG (CDG-Ig)
ALG6	604566	α1-3 Glc-transferase	ALG6-CDG (CDG-Ic)
ALG8	608103	α1-3 Glc-transferase	ALG8-CDG (CDG-Ih)
N-glycosylation			
TUSC3/N33	601385	Oligosaccharyltransferase	Autosomal recessive mental retardation
IAP/MAGT1	300715	Oligosaccharyltransferase	X-linked mental retardation
GCS1	601336	α1-2 glucosidase	GCS1-CDG (CDG-IIb)
MAN1B1	604346	ER α1-2 mannosidase-I	Autosomal recessive mental retardation
MGAT2	602616	β1-2 GlcNAc-transferase	MGAT2-CDG (CDG-IIa)
B4GALT1	137060	β1-4 Gal-transferase	B4GALT1-CDG (CDG-IId)
ST3GAL3	606494	α2-3 Sia-transferase	Autosomal recessive mental retardation
O-GalNAc glycosylation			
GALNT3	601756	polypeptide GalNAc-transferase	Familial tumoral calcinosis
GALNT12	610290	polypeptide GalNAc-transferase	Colorectal cancer susceptibility
COSMC	300611	Core 1 β1-3 Gal-transferase chaperone	Tn syndrome
O-Man glycosylation			
POMT1	607423	Protein O-Man-transferase	Walker-Warburg syndrome
POMT2	607439	Protein O-Man-transferase	Walker-Warburg syndrome

POMGNT1	606822	β1-2 GlcNAc-transferase	Muscle-Eye-Brain disease
LARGE	603590	β1-3 GlcA-transferase/α1-3 Xyl-transferase	MDC1D, limb-girdle muscular dystrophy
FKTN	607440	unknown	Fukuyama congenital muscular dystrophy
FKRP	606596	unknown	MDC1C, limb-girdle muscular dystrophy
O-Fuc glycosylation			
LFNG	602576	β1-3 GlcNAc-transferase	Spondylocostal dysostosis
B3GALTL	610308	β1-3 Glc-transferase	Peters-Plus syndrome
Glycosaminoglycan glycosylation			
XGPT1	604327	Xyl β1-4 Gal-transferase	Ehlers-Danlos syndrome (progeroid type)
B3GAT3	606374	β1-3 GlcA-transferase	Multiple joint dislocations, short stature, craniofacial dysmorphism, and congenital heart defects
EXT1	608177	Heparan sulfate polymerase	Multiple exostoses
EXT2	608210	Heparan sulfate polymerase	Multiple exostoses
CHSY1	608183	Chondroitin synthase 1	Temtamy preaxial brachydactyly syndrome
CHST3	603799	Chondroitin 6-sulfotransferase	Spondyloepiphyseal dysplasia with joint dislocations
CHST14	608429	Dermatan-4-sulfotransferase-1	Ehlers-Danlos syndrome (musculocontractural type)
CHST6	605294	Keratan GlcNAc-6-O-sulfotransferase	Macular corneal dystrophy
Glycolipid glycosylation			
ST3GAL5	604402	α2-3 Sia-transferase (GM3 synthase)	Amish infantile epilepsy syndrome
PIGA	311770	GPI GlcNAc-transferase	Paroxysmal nocturnal

			hemoglobinuria
PIGM	610273	GPI Man-transferase	GPI deficiency
PIGV	610274	GPI Man-transferase	Mabry syndrome
PIGN	606097	GPI ethanolamine-P transferase-1	Multiple congenital anomalies-hypotonia-seizures syndrome
Nucleotide-sugar transport			
SLC35C1	605881	GDP-Fuc transport	SLC35C1-CDG (CDG-IIc)
SLC35A1	605634	CMP-Sia transport	SLC35A1-CDG (CDG-IIf)
SLC35D1	610804	UDP-GlcA/GalNAc transport	Schneckenbecken dysplasia
Vesicular transport			
COG1	606973	Conserved oligomeric Golgi (COG) complex	COG1-CDG (CDG-IIg)
COG4	606976	Conserved oligomeric Golgi (COG) complex	COG4-CDG (CDG-IIj)
COG5	606821	Conserved oligomeric Golgi (COG) complex	COG5-CDG (CDG-IIi)
COG7	606978	Conserved oligomeric Golgi (COG) complex	COG7-CDG (CDG-IIe)
COG8	606979	Conserved oligomeric Golgi (COG) complex	COG8-CDG (CDG-IIh)
SEC23B	610512	COPII subunit (ER to Golgi transport)	Dyserythropoietic congenital anemia type II
ATPV0A2	611716	H+/ATPase, pH regulation in Golgi	Autosomal recessive cutis laxa

Figure 1

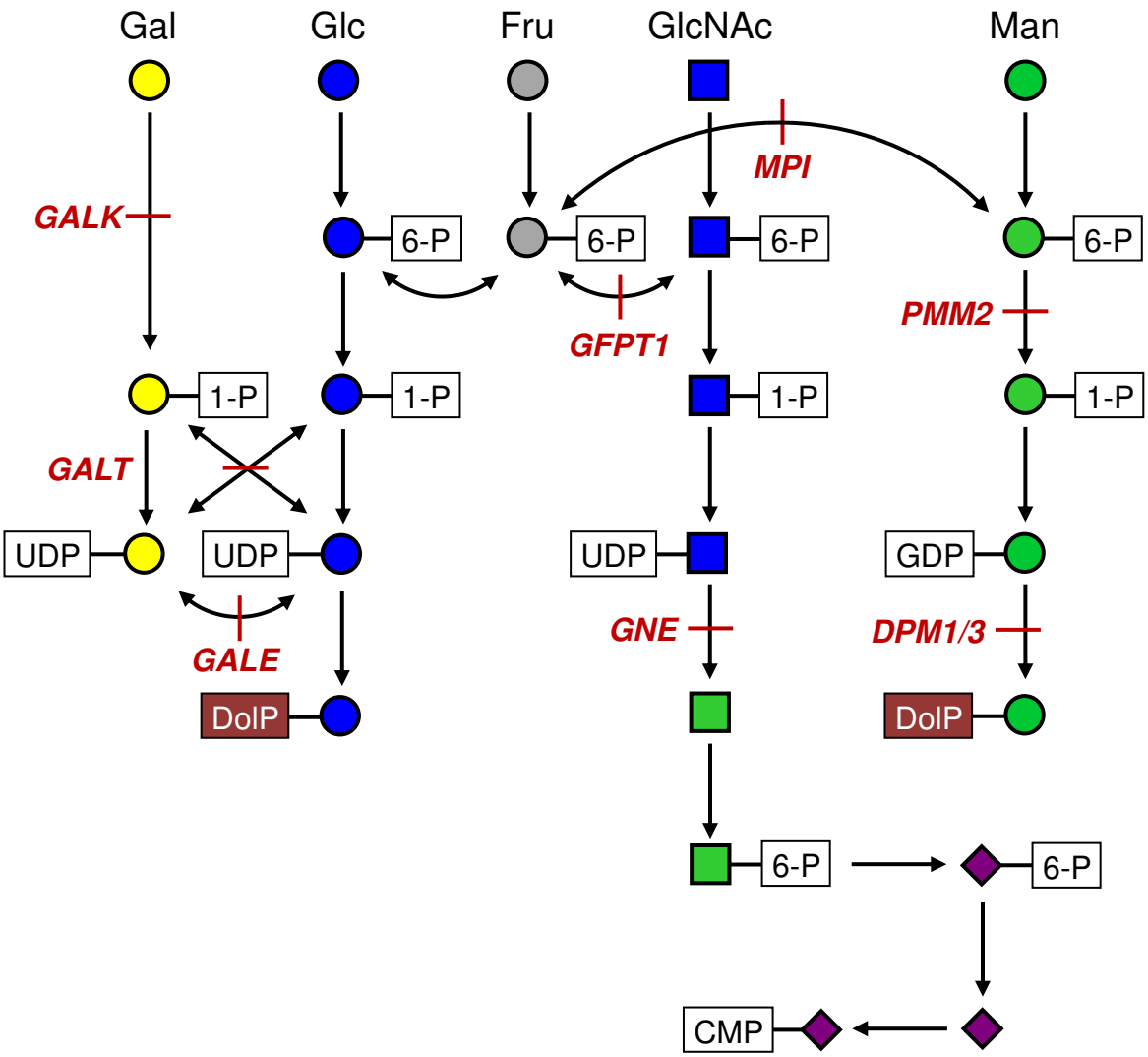

Gal
Glc
Fru
GlcNAc
Man
GALK
MPI
6-P
6-P
6-P
6-P
GFPT1
PMM2
1-P
1-P
1-P
1-P
GALT
UDP
UDP
UDP
GDP
GALE
GNE
DPM1/3
DolP
DolP
6-P
6-P
CMP

Figure 2

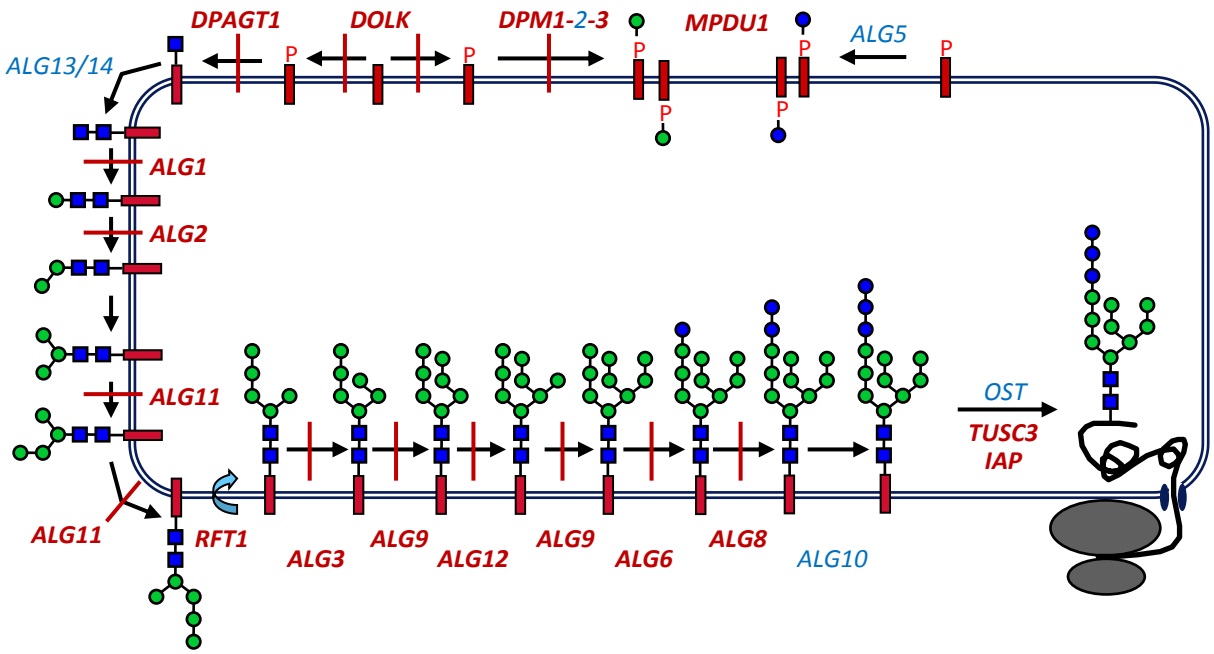
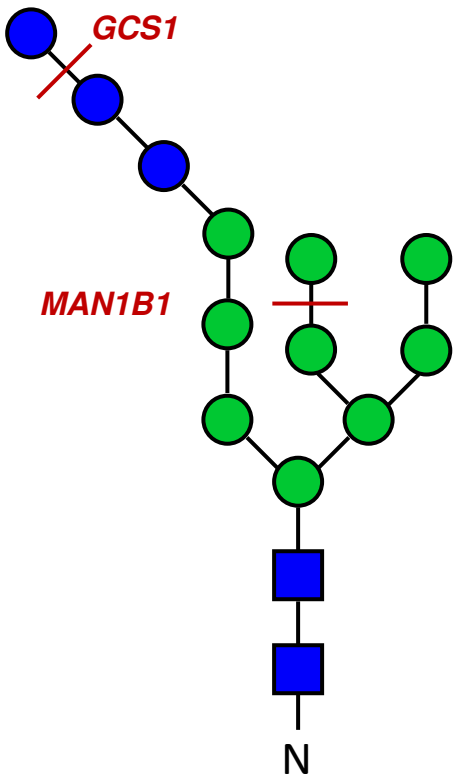
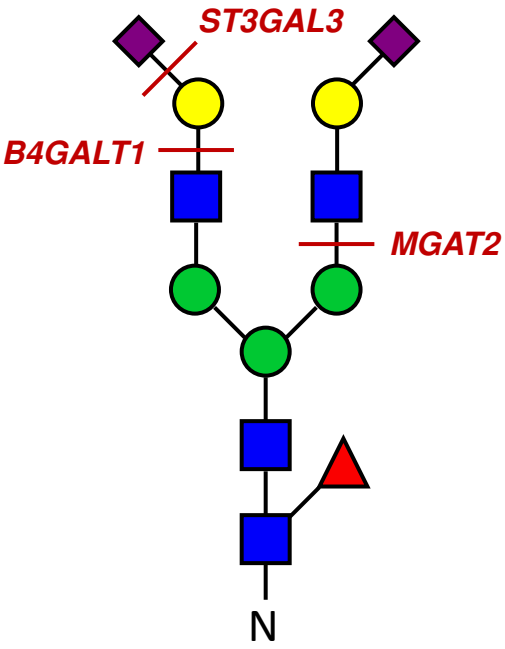

DPAGT1
DOLK
DPM1-2-3
MPDU1
ALG5
ALG13/14
ALG1
ALG2
ALG11
ALG11
RFT1
ALG3
ALG9
ALG12
ALG9
ALG6
ALG8
ALG10
OST
TUSC3
IAP
P

Figure 3

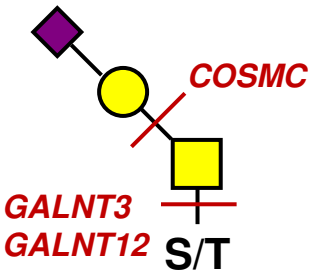


N-glycan (ER)

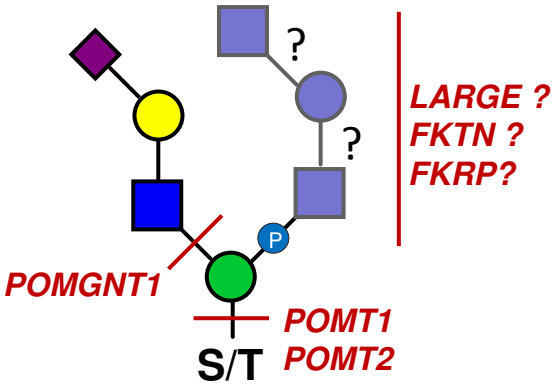


N-glycan (Golgi)

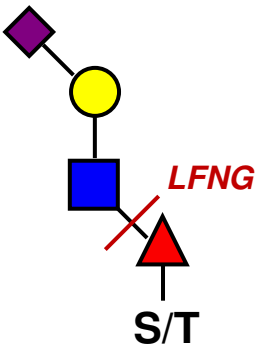
Figure 4



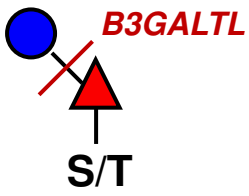
O-GalNAc



O-Man

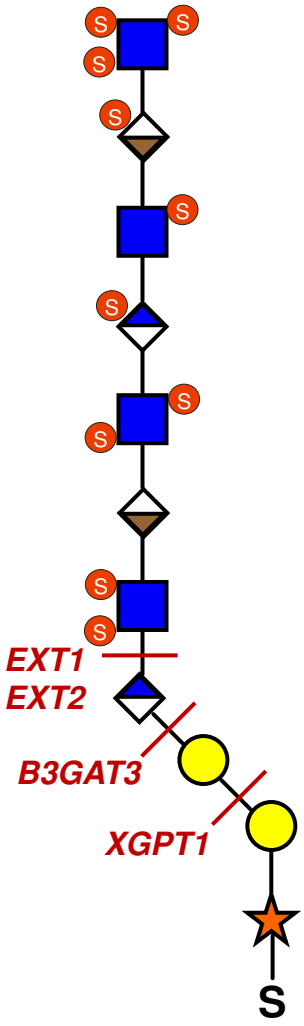


EGF O-Fuc

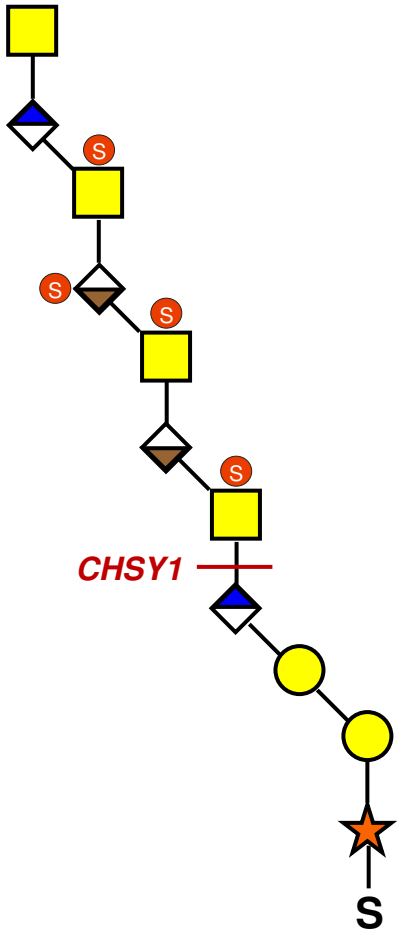


TSP O-Fuc

Figure 5

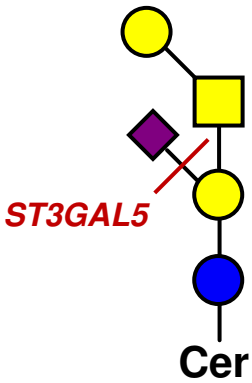


Heparan sulfate

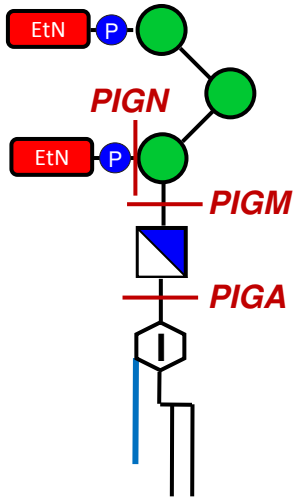


Chondroitin sulfate
Dermatan sulfate

Figure 6



Ganglioside



GPI anchor